(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/30854 A2

(51) International Patent Classification[7]: C07K 16/30, G01N 33/574, A61K 39/00

(21) International Application Number: PCT/SE00/02082

(22) International Filing Date: 26 October 2000 (26.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9903895-2 28 October 1999 (28.10.1999) SE

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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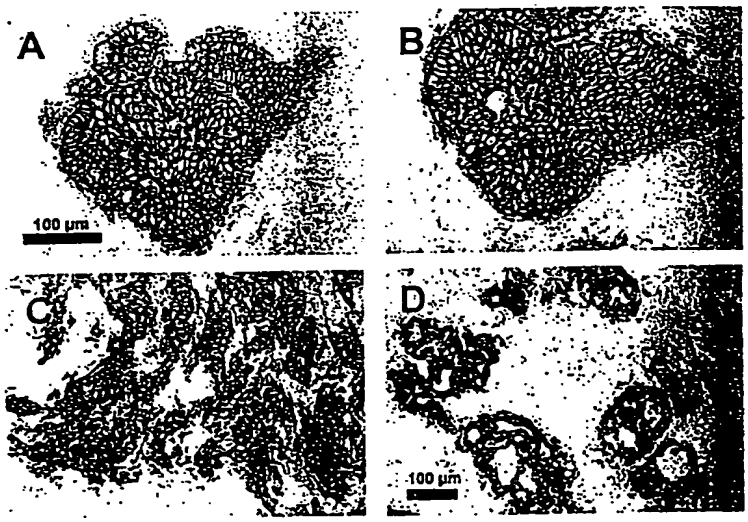
(54) Title: NOVEL COMPOUNDS



(57) Abstract: An antibody, or a derivate or a fragment thereof, having a binding structure for a target structure is described. The antibody is displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells. Said binding structure comprises the complementarity determining region (CDR) sequences in the light chain comprising essentially the amino acids number 23-33 (CDR1), 49-55 (CDR2), 88-98 (CDR3) of the amino acid sequence shown in SEQ ID NO:2, and the CDR sequences in the heavy chain comprising essentially the amino acids number 158-162 (CDR1), 177-193 (CDR2), 226-238 (CDR3) of the amino acid sequence shown in SEQ ID NO:2, or other binding structures with similar unique binding properties. There is also described a target structure displayed in, or on the surface of tumour cells, vaccine compositions, pharmaceutical compositions as well as methods related to human malignant diseases.

WO 01/30854 A2

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

NOVEL COMPOUNDS

The present invention is related to an antibody, or a derivate, or a fragment thereof, having a binding structure for a target structure displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells; and to a target structure displayed in, or on the surface of tumour cells; vaccine compositions; pharmaceutical compositions; as well as methods related to human malignant diseases.

BACKGROUND OF THE INVENTION

Surgery is the primary treatment of colorectal cancer leading to five-year survival rates of 90 to 40 percent depending on the state of tumour progression from Dukes Stage A to C. Conventional adjuvant therapy that includes radiation therapy and chemotherapy has been able to reduce the death rates further by approximately 30 percent (1). Despite these achievements cancer of the colon and rectum is one of the major causes of death in human cancer. Immunological therapy has been extensively attempted. However, colon cancer has generally been resistant to immunotherapy and is considered to be of low immunogenicity. Patients with colon cancer neither respond to IL-2 treatment or adoptive transfer of *in vitro* cultured tumour infiltrating lymphocytes otherwise active in patients with immunogenic malignancies such as melanoma. Most encouraging however, Riethmüller et al. reported a 32 percent decreased seven-year death rate for Dukes Stage C colorectal cancer treated after primary tumour resection with a naked murine mAb directed to a tumour and normal epithelial associated antigen (Ep-CAM) (2), indicating that other immunotherapeutic modalities could be effective.

A significant improvement of adjuvant immunotherapy and of the treatment of more advanced stages of cancer

should require a more potent effector mechanism than provided by a naked mAb. In principle, an increased potency should require an increased tumour selectivity of the targeting antibody.

The limited number of colon cancer associated antigens defined today have been discovered using hybridoma produced murine mAbs resulting from xenogenic immunisations with human tumours (3).

The use of large phage display libraries for the identification of novel tumour-associated antigens can be expected to significantly speed up the process of finding target molecules useful for tumour immunotherapy and diagnosis. Such identification of target molecules could be accomplished by the selection and screening of antibody phage libraries on cultured tumour cells and tissue sections to generate specific reagents defining *in vitro* and *in vivo* expressed antigens (4). The phage display technology has been established as an efficient tool to generate monoclonal antibody reagents to various purified antigens, and the construction and successful selection outcome from immune, naive and synthetic antibody phage libraries have been described in several studies (5).

Non-immune libraries are favourable with respect to their general applicability, making unique libraries for every single target unnecessary. On the other hand, sufficiently large and high quality non-immune libraries are difficult to construct and a target discovery process using these libraries should require efficient subtractive selection methods when based on complex antigens.

A phage library of a more moderate size has now been constructed from a near human primate immunised with complex human antigens. This represents an approach that takes advantage of an in vivo pre-selected repertoire. Such libraries should be enriched for specificities to tumour specific epitopes in a reduced background reactivity to xenogeneic antigens (6). Furthermore, as

compared to the mouse, primate antibodies demonstrating close sequence homology with human antibodies should not be immunogenic in man (7).

Novel primate antibodies from a phage library that define selectively expressed colon cancer associated antigens have now been identified. The therapeutic potential, demonstrated by T cell mediated killing of cultured colon cancer cells coated with two of these antibodies fused to engineered superantigens, is comparable with superantigens fused to murine Fab fragment specific for colon cancer associated antigens such as EP-CAM, for which there has previously been established the therapeutic capacity in experimental systems (8).

There is also provided a method for efficient positive and subtractive cell selection of phage antibodies that should facilitate future identification of novel phenotype specific antigens including tumour associated antigens using antibodies from large phage libraries.

BRIEF SUMMARY OF THE INVENTION

The present invention is related in a first aspect to an antibody, or a derivative or a fragment thereof, having a binding structure for a target structure displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells, said binding structure comprising the complementarity determining region (CDR) sequences in the light chain comprising essentially the amino acids number 23-33 (CDR1), 49-55 (CDR2), 88-98 (CDR3) of the amino acid sequence shown in SEQ ID NO:2, and the CDR sequences in the heavy chain comprising essentially the amino acids number 158-162 (CDR1), 177-193 (CDR2), 226-238 (CDR3) of the amino acid sequence shown in NO: 2, or other binding structures with similar unique binding properties.

In one embodiment the antibody is phage selected. In another embodiment the sequences are of Macaca fascicularis origin. A further embodiment of the invention is a derivative of said antibody, which derivative is of human origin. The sequences preferably have an identity of at least 84% to corresponding sequences of human origin. Preferably, the antibody has low immunogenicity or non-immunogenicity in humans.

In a further embodiment, the antibody has been derivatised by genetically linking to other polypeptides, and/or by chemical conjugation to organic or non-organic chemical molecules, and/or by di-, oligo- or multimerisation.

In still a further embodiment, said antibody is genetically linked or chemically conjugated to cytotoxic polypeptides or to cytotoxic organic or non-organic chemical molecules.

In a further embodiment, said antibody is genetically linked or chemically conjugated to biologically active molecules.

In still a further embodiment, said antibody is genetically linked or chemically conjugated to immune activating molecules.

In another embodiment, said antibody has been changed to increase or decrease the avidity and/or affinity thereof.

In still another embodiment, said antibody has been changed to increase the production yield thereof.

In a further embodiement, said antibody has been changed to influence the pharmacokinetic properties thereof.

In still a further embodiment, said antibody has been changed to give new pharmacokinetic properties thereto.

In a further embodiment, said antibody is labeled and the binding thereof is inhibited by an unlabeled form of said antibody and not by other binding structures, and

not inhibiting the binding of other binding structures having other specificities.

A further embodiment is an antibody, the binding structure of which recognizes a non-reduced form of α6β4 integrin.

In another aspect the invention relates to a target structure displayed in, or on the surface of, tumour cells, said target structure

a) having the ability of being specifically blocked by and to specifically block the binding structure of an antibody as defined in any one of claims 1-14, and other binding structures with similar binding specificities,

b) being displayed in, and on the surface of, human gastrointestinal epithelial cells,

c) having substantial homology with α6 and/or β4 integrin chains or variants thereof, representing a shared or unique epitope,

d) being highly expressed on the surface of tumour cells, and

e) being a target for cytotoxic effector mechanisms.

By substantial homology in this context is meant homology in those parts of the target structure which are relevant for the binding of the antibody.

In one embodiment of said target structure, the binding structure is labeled and the binding thereof is inhibited by an unlabeled form of said binding structure and not by other binding structures, and not inhibiting the binding of other binding structures having other binding specificies.

In a further embodiment of said target structure said binding structure comprises one or more of the complementarity determining region (CDR) sequences comprising essentially the amino acids number 23-33, 49-55, 88-98, 158-162, 177-193, 226-238 of the amino acid sequence shown in SEQ ID NO:2, or other binding structures with similar unique binding properties.

In still a further embodiment of said target structure said binding structure is an antibody, which antibody in a further embodiment comprises the variable region of a light chain comprising essentially the amino acids number 1-109 of the amino acid sequence shown in SEQ ID NO:2, and the variable region of a heavy chain comprising essentially the amino acids number 128-249 of the amino acid sequence shown in SEQ ID NO: 2.

Said target structure is in a further embodiment expressed homogenously in human colonic epithelial cells and less in pancreatic duct and bile duct cells.

In still a further embodiment, the expression of said target structure is correlated to gastrointestinal epithelial differentiation.

In another embodiment, said target structure comprises the amino acid sequence of α6β4 integrin, of which the α6 part is shown in SEQ ID NO: 3 and the β4 part is shown in SEQ ID NO: 4. Another embodiment of the target structure comprises homo- or heteromonomers or homo- or heteromultimers of said α6β4 integrin and/or of said one or more fragments and/or variants and/or subunits thereof. Preferably, said target structure has an apparent molecular weight in its non-reduced form of from 90 to 140 kDa, most preferred fro 80 to 160 kDa.

In still further embodiments the target structure compirses a peptide or polypeptide(s) comprising essentially any one of the amino acid sequences shown in SEQ ID NOs: 5-51, or comprises a molecule complexed to said polypeptide(s).

In the case of a target structure comprising amino acid sequences from the α6β4 integrin, said target structure may in a further embodiment be recognised, exclusively or not, in its non-reduced form by the binding structure comprised by the antibody as defined above.

The invention relates in a further aspect to a substance which binds to the target structure as defined

above, which substance is an organic chemnical molecule or a peptide. In one embodiment, said substance is an anti-idiotype of said target structure. Said anti-idiotype may be specifically blocked by and specifically block a binding structure having similar binding specificity for said target structure.

In a still further aspect, the invention relates to a substance that blocks the function of the target structure as defined above, which substance is an organic molecule or a peptide.

In another aspect, the invention relates to a binding structure which recognises a target structure as defined above and which is of an organic chemical nature.

In a further aspect, the invention relates to a pharmaceutical compositin comprising as an active principle an antibody as defined above, or a target structure as defined above, or a substance as defined above.

In still a further aspect, the invention is related to a vaccine composition comprising as an active principle abn antibody as defined above, or a target structure as defined above, or a substance as defined above.

In a further aspect, the invention is related to a method of therapy for treating conditions based on an anti-angiotenic mechanism, whereby an antibody as defined above, or a target structure as defined above, or a substance as defined above, is administered to a human subject.

In another aspect, the invention is related to a method of treating human metastatic diseases, wherein an antibody as defined above is administered to a human subject.

In a further aspect the invention is related to a method of in vitro histopathological diagnosis and prognosis of human malignant disease, whereby a sample is

contacted with an antibody as defined above and an indicator.

Embodiments of said method comprise tumour typing, tumour screening, tumour diagnosis and prognosis, and monitoring premalignant conditions.

In still a further aspect, the invention is related to a method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of an antigen comprising a target structure, as defined above, or an anti-idiotype of said target structure, as defined above, is assayed.

A further aspect of the invention is related to a method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of an antibody as defined above is assayed.

A still further aspect of the invention is related to a method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of a complex of a) an antigen comprising a target structure, as defined above, or an anti-idiotype of said target structure, as defined above, and b) an antibody, as defined above, is assayed.

In a still further aspect, the invention is related to a method for in vivo diagnosis and prognosis of human malignant disease, whereby the localisation of an antibody, as defined above, to tumour deposits in a human subject is determined. Said antibody is preferably administered to the subject before the determination. In one embodiment said antibody is accumulated in tumour deposits. In a further embodiment, said method is quantitative.

Another aspect of the invention is related to a method for therapy of human malignant disease, whereby an antibody, as defined above, is administered to a human subject. In one embodiment of this method said antibody has been changed by being genetically linked to molecules giving the combined molecule changed pharmacokinetic

properties. In another embodiment said antibody has been changed by being derivatised.

DETAILED DESCRIPTION OF THE INVENTION

The identification of novel tumour associated antigens (TAAs) is pivotal for the progression in the fields of tumour immunotherapy and diagnosis. In relation to the present invention, there was first developed, based on flow cytometric evaluation and use of a mini-library composed of specific antibody clones linked to different antibiotic resistance markers, methods for positive and subtractive selection of phage antibodies employing intact cells as the antigen source. An scFv phage library (2.7×10^7) was constructed from a primate (*Macaca fascicularis*) immunised with pooled human colon carcinomas. This library was selected for three rounds by binding to Colo205 colon adenocarcinoma cells, and proteolytic elution followed by phage amplification.

Several antibodies reactive with colon carcinomas and with restricted reactivity with a few epithelial normal tissues were identified by immunohistochemistry. One clone, A3 scFv, recognised an epitope that was homogeneously expressed in 11/11 of colon and 4/4 pancreatic carcinomas studied and normal tissue expression restricted to subtypes of epithelia in the gastrointestinal tract. The A3 scFv had an apparent overall affinity about 100-fold higher than an A3 Fab, indicating binding of scFv homodimers. The cell surface density of the A3 epitope, calculated on the basis of Fab binding, was exceptionally high, approaching 3 million per cell.

Efficient T cell mediated killing of colon cancer cells coated with A3 scFv fused to the low MHC class II binding superantigen mutant SEA(D227A) is also demonstrated. The identified A3 molecule thus represents a TAA with properties that suggests its use for immuno-therapy of colon and pancreatic cancer.

DISCUSSION

In relation to the present invention, efficient protocols for phage selection to be used for the identification of cell phenotype specific antibody fragments from large phage libraries was developed. The target specificities for the applications as exemplified were for colon tumour associated antigens.

First the frequency of pIII-scFv fusion protein surface display in the phage population using the herein presented phagemid construct for phage propagation was analysed. A higher level of C215 scFv display was achieved as compared to previous reports. This should favour subtractive selection efficiency, but also increases the probability of avidity selection of low affinity antibodies from libraries.

Specificity of C215 scFv phage binding to colon adenocarcinoma Colo205 cells was clearly demonstrated. Bound phage could be efficiently eluted by use of the protease Genenase that specifically cleaves a target sequence between the phage protein III and the scFv antibody leaving the cells intact after elution. This non-chemical elution method should equally efficiently elute phage antibodies irrespectively of their binding affinity and only phage bound by scFv interactions, adding to the specificity of the process.

The enrichment achieved after three selection rounds on Colo205 cells (500 000x) using this selection protocol was similar to that reported by other investigators for selections on complex antigens.

After verifying the performance of the various methodological steps the combined technology was applied to library selections using Colo205 cells.

The library was constructed from a near human species immunised with human tumours. The antibody pool generated this way would potentially include affinity matured antibodies to tumour specific antigens in a limited background of xeno reactivities to widespread

normal human tissue antigens (6). The antibodies identified recognised tumour and tissue differentiation antigens with restricted normal tissue distribution. All of the selected antibodies identified as colon cancer tissue reactive in the primary screening also reacted with viable Colo205 cells in flow cytometry. This restriction to cell surface specificities should reflect the selection process and not the composition of the library, since a suspension of a mixture of tumour tissue components was used for the immunisation.

In a similar previous study extra- and intracellular specificities were identified in an anti-melanoma library produced the same way and selected using tissue sections as the antigen source (4). Tissue sections of resected human colorectal tumours and normal colon (mounted in the same well) were used for the primary screening using immunohistochemistry to assure the clinical relevance of the selected specificities, to increase the efficiency and to obtain more qualitative information as compared to flow cytometric screening.

The selected antibodies could be classified into four antibody specificity groups, distinguished by their reactivity patterns to epithelia in different organs (see Example 1, Table 1). Among these specificity groups, A3 scFv identified the most tumour selective antigen. This A3 TAA was highly, homogeneously and frequently expressed in samples of primary and metastatic colon cancer and of pancreatic cancer. Furthermore, its cell surface expression level as determined with the A3 Fab fusion protein (3 millions epitopes/cell) was exceptionally high and permissive for cell surface mediated cytotoxic effects.

Few, if any, of the frequently expressed human tumour antigens defined are tumour specific, but are commonly related to tissue differentiation such as A3 and the Ep-CAM. However, upregulated expression of these antigens in tumours should provide a basis for a

therapeutically active dose window. The availability from the circulation of normal tissue compartments expressing the antigen may also be more restricted due to limited capillary permeability and their site of expression in the body (e.g. the exposure of the apical side of gut epithelial cells to circulating antibodies should be very limited).

The clinical experience with the pan-epithelial Ep-CAM reactive 17-1A mAb supports the feasibility to identify an effective non-toxic antibody dose. The restricted expression in epithelia of all of the selected scFv clones in this work, indicate that these clones in principal could be evaluated as candidates for immuno-therapeutic applications analogously to the 17-1A, e.g. as full-length mAbs. However, a particular advantage for the A3 TAA as compared to the Ep-CAM is the lack of expression in most normal epithelia such as of the lung and kidney, although the expression in the colon is similar.

The tissue distribution to subtypes of normal epithelia is supported by the selective expression in subtypes of carcinomas originating from the gastro-intestinal tract (see Example 2, Table 2).

Several of the previously well-known colon cancer associated antigens (CEA, CA50, CA19-9, CA242, Tag-72)(3) are expressed equally or more restrictedly in normal tissues as compared to the A3 epitope. However, in contrast to the A3 and the C215 Ep-CAM they are more heterogeneously expressed in tumours.

Use of antibodies to the Ep-CAM has demonstrated good clinical results including a survival advantage for colorectal cancer patients in an adjuvant setting (2). With the objective to induce tumour responses even in more advanced stage patients, the introduction of potent effector molecules in conjunction with this antibody will challenge the "normal tissue resistance" seen in the treatment with the naked 17-1A mAb. Preclinically, this

could be studied in model systems using toxin-conjugated antibodies specific to the murine version of this antigen or animals transgenic for human colon cancer associated antigens.

Previously, antibody immunotoxins have been successfully used to cure mice in models with metastatically growing tumours expressing xeno (human) tumour antigens not expressed in mouse tissues (10). However, the TAAs used are truly tumour specific and the models do not reflect the potential for normal tissue targeted toxicity.

In previous studies we have reported the potential of superantigens as immunostimulatory toxins for tumour immunotherapy (8). Antibody mediated targeting of superantigens attracted large numbers of cytotoxic and cytokine-producing T cells to the tumour site. The superantigen SEA(D227A), mutated for low MHC class II binding affinity, was genetically linked to tumour targeting antibodies. This "tumour-selective" agent was applied to recruit T cells independent of MHC expression in the tumour, thus short-cutting the problems of MHC down regulation and polymorphism that represent significant obstacles for other active immunotherapeutic approaches.

The mini-library of the established "tumour-selective", 1F scFv phage, the "broadly-reactive" C215 phage and the non-specific D1.3 phage antibody clones was an essential tool for the development of protocols for efficient subtractive cell selection. A requirement for this selection principle is that the negative selection is followed by positive selection before phage rescue and amplification, due to the high frequency of non-displaying phage particles. Alternatively, non-displaying phage can be made non-infective by selective proteolysis (G. Winter, pers. comm.). Such a technique may allow the generation of "inert libraries", i.e. libraries that have been extensively negatively preselected (e.g. towards a

cell in a resting state or a transfectable parental cell).

In conclusion, the "non-wanted" model phage specificity could selectively be subtracted from the phage population by a factor of approx. 100 for each selection round. Future subtractive selections using the developed protocol in combination with the use of large non-immune phage libraries for identification of differentially expressed cell surface antigens will demonstrate whether such an approach prove to be superior to the strategy we used in this study, i.e. positive selection using an in vivo pre-selected immune repertoire, including restrictions and biases such as immunodominance (4). The low affinity and high epitope density demonstrated for the A3 Fab binding to tumour cells as compared to the A3 scFv fusion protein suggests formation of scFv multimers that interact with epitopes that cluster on cell surfaces. Higher affinity monovalent variants of A3 Fab or alternatively, stable divalent constructs such as full-length A3 Fv grafted mAbs compatible with the putative low immunogenicity of A3 should be developed. Such constructs would be suitable for targeting of appropriate effector molecules to this highly expressed gastro-intestinal tumour associated antigen.

The invention is further illustrated in the following nonlimiting experimental part of the description.

EXMPERIMENTAL PART

Materials and Methods

Animals

Cynomolgus Macaque (*Macaca fascicularis*) monkeys were kept and immunised at the Swedish Institute for Infectious Decease Control (SIIDC), Stockholm. Water and food w re always available *ad libitum*. Four monkeys were immunised subcutaneously with 2 ml of a crude suspension of colon cancer tissues in 10 % normal cynomolgus serum in PBS. Booster doses were given day 21, 35, and 49.

Antibody responses were demonstrated in two monkeys where the antigen had been admixed with alum adjuvant. All animals were kept according to Swedish legislation and the experiments were approved by the local ethical committees.

Tissues and cells

Human tumours and normal tissue samples were obtained from Lund University Hospital and Malmö General Hospital, Sweden. The human colorectal cell line Colo205, the human B cell lymphoma cell line Raji and the murine B16 melanoma cell line were from the American Tissue Culture Collection (ATCC, Rockville, MD). The mouse melanoma B16-C215$^+$ cells transfected with the expression vector pKGE839 containing the Ep-CAM-1 gene (C215) has been described previously (9).

The human cells were cultured in RPMI 1640 medium (Gibco, Middlesex, UK) supplemented with 10% heat inactivated foetal bovine serum (Gibco) and 0.1 mg/ml gentamycin sulphate (Biological Industries, Kibbutz Beit Haemek, Israel). The mouse cells were cultured in medium additionally supplemented with 1 mM glutamine (Hyclone, Cramlington, UK), 5×10^{-5} M β-mercaptoethanol (ICN, Costa Mesa, CA), 0.2 % $NaHCO_3$ (Seromed Biochrome, Berlin, Germany), 1×10^{-2} M HEPES (HyClone, UT) and 1×10^{-3} M sodium pyrovate (HyClone). The cells were repeatedly tested for Mycoplasma contamination with Gene-Probe Mycoplasma T. C. test (San Diego, CA).

Phagemid vector and phage library construction

Total spleen RNA was extracted from one of the responding monkeys using an RNA isolation kit from Promega (Mannheim, Germany) and cDNA was amplified using an RNA PCR kit from PE Biosystems (Stockholm, Sweden). The primers for cDNA synthesis of lambda light chain and heavy chain genes and for the assembly of these genes to scFv genes have been reported previously (4). The scFv cDNA was ligated into a phagemid vector (4) in fusion with the residues 249-406 of the M13 gene III. The scFv-

gIII gene was expressed from a *pho*A promoter and the resulting protein was directed by the *E. coli* heat stable toxin II signal peptide.

Repeated electroporations of 7 μg library vector with scFv gene inserts resulted in a total of 2.7×10^7 primary transformed *E. coli* TG-1 growing as colonies on minimal agar plates. The colonies were scraped from the plates and grown in 2xYT at 150 rpm and 37°C for 1h. The culture was superinfected with M13K07 helper phage (Promega) in 50 times excess. Ampicillin to a concentration of 100 mg/l was added and the culture grown for a further hour. After addition of kanamycin to a concentration of 70 mg/l, the culture was grown for 15 h at 30°C and 250 rpm. The phage particles were harvested from the culture supernatant using two repeated PEG/NaCl precipitations. The precipitated phage was resolved in PBS 1% BSA.

Western blot analysis

A two-fold dilution series of scFv-C215 phage particles (from an undiluted stock of PEG-precipitated/concentrated phage) was applied to separation on a reducing 12% polyacrylamide gel with 1% SDS and 2% β-mercaptoethanol. The proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) by electrophoresis. The membrane was blocked with 5% low-fat milk (Semper AB, Stockholm, Sweden) and then incubated with a rabbit antiserum against a protein III derived peptide sequence, AEGDDPAKAAFNSLQASATEC, conjugated to keyhole limpet hemocyanin. Secondary horse radish peroxidase (HRP) conjugated goat-anti-rabbit antibodies (Bio-Rad) were incubated for 30 min. Between all steps the membrane was washed 3 times during 5 min in PBS/ 0.5% Tween 20. The membrane was incubated in substrate (Amersham Pharmacia Biotech, Little Chalfon Buckinghamshire, UK) for one min. A light sensitive film (ECL hyperfilm, Amersham) was exposed to the membrane and developed for 0.5-5 min.

Similarly, to analyse the integrity of purified Fab (A3, including cynomolgus CH1 and Clambda domains), scFv- and Fab (including murine CH1 and Ckappa)-SEA(D227A) fusion proteins (produced as described previously (9)), 12% SDS-PAGEs were performed. The membranes with transferred proteins were incubated with purified polyclonal rabbit anti-SEA antibodies followed by the reagent steps described above.

Model and library phage selection on cells

Phage suspensions of the lambda light chain library (or of model phage), 10^{12} in 100 µl PBS/1% BSA, were incubated with 3 million Colo205 cells for 1h on ice. The cells were washed 3 times including a 10-min incubation using 2 ml PBS/1% BSA for each wash. The phage were eluted by adding 50 µl of 33 µg/ml Genenase to the cell pellet and incubated for 15 min. Genenase, which is a subtilisin BPN´ mutant, S24C/H64A/E156S/G169A/Y217L, was kindly provided by Dr. Poul Carter (San Francisco, CA). After centrifugation the supernatant was transferred to a new tube and 250 µl 1% BSA in PBS was added. To rescue and amplify the selected library (and the model phage particles in the multi-pass experiment), the eluted phage particles were allowed to infect 1 ml, *E. coli* DH5αF´ ($OD_{600\ nm}$ = 1.0). The infected bacterial culture was diluted 100 times with 2×YT supplemented with the proper antibiotic and cultured until an OD >1.0 (up to two days).

Finally, to produce soluble scFv the amber suppressor strain HB2151 of *E. coli* was infected with the selected library from the second and third round. After growth on agar plates containing ampicillin, single colonies were cultured in 96 Micro well plates in 2×YT medium supplemented with ampicillin at 30°C for 17 h. After centrifugation, removal of the supernatant to which an equal volume of PBS/1%BSA was added, individual scFvs were analysed for immunoreactivity to sections of human tumours and normal tissues. Briefly, the C-terminal tag,

ATPAKSE, was detected using a rabbit antiserum followed by biotinylated goat anti-rabbit antibodies (DAKO A/S, Copenhagen, Denmark) and StreptABComplex HRP (DAKO A/S) (see "Immunohistochemistry").

Immunohistochemistry

Frozen cryosections (8 μm) were air-dried on slides, fixed in acetone at -20°C for 10 min and rehydrated in 20% foetal bovine serum in PBS (FBS). Endogenous biotin was blocked with avidin (diluted 1/6) for 15 min and then with biotin (diluted 1/6) for 15 min (Vector Laboratories, Burlingame, CA). Affinity purified and biotinylated rabbit anti-SEA antibodies, 5 μg/ml, were incubated for 30 min followed by StreptABComplex HRP (DAKO A/S, Copenhagen, Denmark), 1/110 diluted in 50 mM Tris pH 7.6 for 30 min. Between all steps the sections were washed 3 times in TBS. The staining reaction was developed for 8 min in 0.5 mg/ml 3,3´-diaminobenzidine tetrahydrochloride (Sigma) dissolved in Tris pH 7.6 with 0.01 percent H_2O_2. After 10 min counterstaining in 0.5% methyl green, the slides were rinsed for 10 min in tap water and gradually dehydrated in 70-99% ethanol and xylene before mounting in DPX medium (Sigma).

Flow cytometry

The Colo205 colon cancer cells were dissociated with 0.02% w/v EDTA and washed with PBS. To follow the development of an antibody response in the monkeys the cells were incubated consecutively with diluted serum, for 1h at 4°C, biotinylated rabbit anti-human IgG antibodies (Southern Biotechnology Ass. Inc., Al, USA) for 30 min, and finally with avidin-PE (Becton Dickinson, Mountain View, CA) for 30 min.

The binding of model phage to the cells was analysed using rabbit-anti-M13 antibodies (produced by immunisation of rabbits with M13 particles) and FITC conjugated donkey anti-rabbit antibodies (Amersham Pharmacia Biotech). The binding of antibodies fused to SEA(D227A) was detected using biotinylated rabbit anti-

SEA antibodies and avidin-PE. All reagents were diluted in PBS/1% BSA. The cells were washed twice with PBS/1% BSA after incubations with reagents and three times including 10 min incubations after binding of phage particles.

Flow cytometric analysis was performed using a FACSort flow cytometer (Becton Dickinson).

Affinity determination on cultured cells

A3 scFv-SEA(D227A), A3 Fab-SEA(D227A) and 1F scFv SEA(D227A) fusion proteins, 80 μg of each protein, were labelled with iodine as described by Bolton and Hunter to a specific activity of 10-15 μCi/μg. Colo205 cells and Raji cells, 30 000/sample were incubated with the iodinated fusion protein at 100 μl/tube in a two-fold dilution series in 1% BSA for 1h and then washed three times in PBS before measuring bound activity. The concentration of added and bound fusion protein was used for Scatchard analysis. The background binding to the Raji cells was subtracted to calculate the specific binding to the Colo205 cells.

Cytotoxicity assay

The T cell dependent cytotoxicity of the superantigen fusion protein (superantigen antibody dependent cellular cytotoxicity, SADCC) was measured in a standard 4 h chromium-release assay employing ^{51}Cr-labelled Colo205 cells as target cells and human T cells as effector cells (9). The percent specific lysis was calculated as:

$$100 \times \frac{\text{cpm experimental release - cpm background release}}{\text{cpm total release - cpm background release}}$$

EXAMPLE 1

Generation of tumour binding monoclonal cynomolgus antibodies

Cynomolgus monkeys, *Macaca fascicularis* (four individuals) were repeatedly immunised with a suspension

of human colon carcinomas four times every other week. The gradual development of an antibody response in the monkeys was followed by flow cytometric staining of cultured colorectal cells, Colo205, using dilution series of the preimmune and immune sera. An IgG antibody response was elicited only when alum precipitated tumour tissue suspensions were used (two individuals).

The monkey with the highest binding level of immune to preimmune serum antibodies was used for the construction of a large combinatorial scFv phage library of approximately 2.7×10^7 (estimated from the number of primary transformants). The primate phage library was selected using Colo205 cells. The total phage yield (eluted/added number of phage counted as colony forming units, CFU) from three consecutive selection rounds increased gradually from 1.9×10^{-7}, 1.4×10^{-5}, to 1.2×10^{-3}. Five percent (12/246) of the monoclonal soluble scFv:s produced from the phage library after the third round of selection were demonstrated to bind to sections of a human colorectal cancer tissue and to intact Colo205 cells by flow cytometry. All of the selected antibodies demonstrated individually unique nucleic acid sequences according to Hinf I restriction patterns analysed by 1% agarose gel electrophoresis.

The antibody genes were amplified by polymerase chain reaction using 5 µl of bacterial cultures and primers complementary to regions 5´and 3´ to the scFv gene in the phagemid vector (regions in the *pho*A promoter and in the M13 gene III).

The selected scFv demonstrate individually unique reactivity with epithelia in normal tissues

The colorectal cancer reactive scFv's were classified into specificity groups based on their immunohistochemical reactivity pattern with normal tissues (Table 1). The antibodies studied in detail were A3 scFv (and A3 scFv-SEA(D227A)), A10 scFv, 3D scFv and 1D scFv. The representative antibodies could be

distinguished from each other by their fine specificity to epithelia in different organs and by their binding to leukocytes. The 1D scFv strongly reacted with gut epithelia and was the only antibody that reacted with cells of polymorph nuclear granulocyte morphology. The 1D scFv also differed from the other antibodies by staining the luminal surface of kidney tubuli and collecting ducts whereas the A10 scFv reacted homogeneously (non-polarly) with these epithelial cells and 3D scFv and A3 scFv were negative. 1D, A10 and 3D, but not A3 scFv also reacted with macrophage-like cells in the lung.

A fifth group of antibodies, not extensively evaluated and thus not included in Table 1, reacted with colon epithelia, leukocytes and Kuppfer cells in the liver. The A3 scFv stands out as demonstrating the most restricted reactivity with the panel of normal tissues used. The most prominent normal tissue reactivity of the A3 was staining of normal colon epithelium. Weak staining were also detected in small ducts of the pancreas and bile ducts of the liver and of substructures in small bowel epithelia. The surface epithelium of one of the two stomach samples was strongly stained by the A3 antibody.

The reactivity pattern of the A3 scFv was confirmed using the fusion protein A3 scFv-SEA(D227A). This format permitted the use of polyclonal rabbit anti-SEA antibodies for immunohistochemical detection, which is a more sensitive detection system demonstrating lower background and tissue crossreactivity as compared to the use of secondary antibodies to the peptide tag, ATPAKSE, at the C-terminus of the scFvs.

Table 1 Immunohistochemical reactivity to normal human tissues of soluble scFv fragments from the selected colorectal cancer phage library

		scFv clone designation			
Tissue / sub-structure	n*	A3 **	A10	3D	1D
Esophagus / epithelial tissue	1	0	ND	ND	ND
/ non-epithelial tissue		0	ND	ND	ND
Colon / epithelium	5	++	+	+	++
/ non-epithelial tissue		0	0	0	granulocytes ++
Small bowel / villi epithelium	2	(+)	heterogenously +	+	heterogenously (+)
/ basal glandulae		+	+	+	++
/ non-epithelial tissue		0	0	0	0
Ventricle / surface epithelium	2	0, ++	0	0, +	++
/ glandular epithelium		0	+, ++	0	++
/ non-epithelial tissue		0	0	0	0
Pancreas / acini	1	0	(+)	+	++
/ small ducts		(+)	(+)	+	++
/ large ducts		0	(+)	+	++
/ non-epithelial tissue		0	0	0	0
/ endocrine		0	0	0	0
Liver / hepatocytes	2	0	ND	ND	ND
/ Kuppfer cells		0	ND	ND	ND
/ bile ducts		(+)	ND	ND	ND
Kidney / proximal tubuli	1	0	+	0	luminal surface ++
/ distal tubuli		0	+	0	luminal surface ++
/ collecting ducts		0	+	0	luminal surface ++
/ glomeruli		0	0	0	0
/ non-epithelial tissue		0	0	0	0
Bladder / epithelial tissue	1	0	ND	ND	ND
/ non-epithelial tissue		0	ND	ND	ND
Prostate / epithelial tissue	1	0	++	+	and secreted material ++
/ non-epithelial tissue		0	0	0	0
Lung / bronchial epithelium	1	0	(+)	(+)	0
/ alveolar epithelium		0	(+)	(+)	0
/ non-epithelial tissue		0	macrophages +	macrophages +	granulocytes ++, macrophages +
CNS / gray matter	1	0	ND	ND	ND
/ white matter		0	ND	ND	ND
Skeletal muscle	1	0	ND	ND	ND

Notes to Table 1

0 = negative, (+) = weak, + = moderate, ++ = strong, ND = not determined

* Number of tissue samples examined

** The reactivity of A3 scFv has been confirmed with the A3 scFv SEA(D227A) fusion protein

EXAMPLE 2

The A3 tumour-associated antigen is homogeneously and frequently expressed in colorectal and pancreatic tumours

The A3 scFv-SEA(D227A) fusion protein was used for immunohistochemical staining of various tumours of epithelial origin (Table 2 and Figure 1). The fusion protein homogeneously and strongly stained 11/11 samples of primary colon cancer tissues and 4/4 metastatic colon cancer samples resected from the ovary, a lymph node and the liver. Pancreatic cancer tumours, 4/4 samples, were equally strongly positive. In contrast, tissue samples of gastric, prostate, breast and non-small cell lung carcinomas were negative.

Table 2 Tumor tissue reactivity of A3 scFv SEA(D227A)

Tumor tissue	n	Reactivity
Colon cancer, primary tumors	11	All tumor cells are strongly and homogenously stained
Colon cancer metastasis in lymph node, liver and ovary	4	As above
Pancreas cancer	4	As above
Ventricle cancer	2	Negative
Prostate cancer	2	Negative
Breast cancer	2	Negative
Lung carcinoma (non-small cell)	2	Negative
Malignant melanoma	2	Negative

EXAMPLE 3

The A3 TAA is highly expressed on the surface of colon cancer cells

The results from several Scatchard plots for affinity determination, based on the binding of the fusion proteins A3 scFv-SEA(D227A), A3 Fab and 1F scFv-SEA(D227A) (1F was classified to the A3 specificity group) to Colo205 cells, are summarised in Table 3. Specific binding was calculated by subtraction of non-specific binding to human B cell lymphoma Raji cells, a cell line not expressing the A3 and 1F TAAs, from the binding to Colo205 cells. Linear regression was used to calculate the slope and the intercept of the extrapolated line in the Scatchard plot. The A3 scFv-SEA(D227A) fusion protein saturated approximately 10-fold less binding sites per cell as compared to the A3 Fab (approx. 3 million sites per cell) fusion protein, indicating that divalent (multivalent) binding was involved for the scFv. This is supported by the more than 100-fold higher overall affinity (3.6-5.5 nM) for the A3 scFv fusion protein as compared to the A3 Fab (580-780 nM).

A single experiment performed using the 1F scFv-SEA(D227A) fusion protein, demonstrated similar binding affinity and saturation of binding sites as the A3 scFv-SEA(D227A) fusion protein.

Table 3 Scatchard analysis of iodinated fusion proteins binding to Colo205 cells

Fusion protein	n*	Kd (nM)	million sites /cell
A3 Fab-SEA(D227A)	2	580-780	3.0-3.9
A3 scFv-SEA(D227A)	3	3.6-5.5	0.11-0.39
1F scFv-SEA(D227A)	1	4.2	0.18

* Number of experiments performed

EXAMPLE 4

A3 and 1F scFv-SEA(D227A) mediate T cell lysis of Colo205 cells

The capacity of the two fusion proteins A3 and 1F scFv-SEA(D227A) to mediate superantigen antibody dependent cellular cytotoxicity (SADCC) towards Colo205 cells was investigated and compared with the positive control C215 Fab-SEA(D227A) and negative control D1.3 scFv-SEA(D227A) fusion proteins. The A3 scFv-SEA(D227A) fusion protein titration approached a plateau for maximal lysis which was similar, approx. 50 percent in this 4 h assay, to that demonstrated for the C215 Fab-SEA(D227A) fusion protein, although at a ten-fold higher concentration (Figure 2). The 1F scFv-SEA(D227A) mediated a similar level of cytotoxicity at a slightly higher concentration as compared to the A3 scFv-SEA(D227A).

The negative control D1.3 scFv SEA(D227A) fusion protein did not mediate any cytotoxicity.

EXAMPLE 5

Purification of a tumour associated antigen that is recognised by the colon cancer reactive antibody A3.

A tumour extract was made out of xenografted tumour cell line Colo205. The extract was applied to a pre-column coupled with C215Fab-SEAm9, and a column coupled with A3scFv-SEAm9. The columns were in series, during the application of sample but separated prior to elution under alkaline conditions.

A single peak was detected during elution by UV spectroscopy (Figure 3). This eluted fraction from the latter A3-column was collected, neutralised, concentrated, and then analysed by SDS-PAGE under non-reducing conditions (Figure 4). Two bands visible by silver staining (labelled I and II in Figure 4) of apparent molecular weight of approximately 90-140 kDa were cut out and examined by standard peptid mapping methodologies. These two bands corresponds to bands detected by A3 in Western Blot, see example 8. From band

I 47 separate tryptic peptide masses were obtained (see SEQ ID NO: 3, Table 4, and Fig 5 for the sequnces and corresponing mass weights) which completely matched to different tryptic peptide masses. as determined by MALDI-TOF) of the human α6 integrin or β4 integrin (see SEQ ID NOs: 5-51 and 3-4, respectively, and Fig 3A and B, respectively, where in Fig 3A the underlinings correspond to the peptides appearing in Fig 3B/SEQ ID NOs: 5-51). From band II 22 separate tryptic peptide masses were obtained which completely matched to different tryptic peptide masses of β4 integrin (data not shown). The data show that the α6β4 integrin heterodimer is specifically isolated with the A3-affinity column.

Table 4 *Peptides/polypeptides derived from human α6β4 integrin and masses thereof*

Sequence No.	Sequence	Measured Mass	Calculated Mass
5	LLLVGAPR	838.568	838.551
6	ANRTGGLYSCDITARGPCTR	2226.131	2226.050
7	VVTCAHRYEK	1262.637	1262.631
8	RQHVNTK	882.524	882.490
9	CYVLSQNLR	1152.618	1152.583
10	FGSCQQGVAATFTK	1501.706	1501.710
11	DFHYIVFGAPGTYNWK	1914.881	1914.917
12	DEITFVSGAPR	1191.625	1191.600
13	ANHSGAVVLLK	1108.600	1108.647
14	DGWQDIVIGAPQYFDR	1879.865	1879.897
15	DGEVGGAVYVYMNQQGR	1842.811	1842.844
16	WNNVKPIR	1026.608	1026.584
17	NIGDINQDGYPDIAVGAPYDDLGK	2520.213	2520.189
18	GISPYFGYSIAGNMDLDR	1975.913	1975.922
19	NSYPDVAVGSLSDSVTIFR	2026.992	2027.008
20	SRPVINIQK	1054.644	1054.637
21	LRPIPITASVEIQEPSSR	1993.066	1993.108
22	VNSLPEVLPILNSDEPK	1863.920	1864.006
23	TAHIDVHFLK	1180.665	1180.647
24	FSYLPIQK	995.601	995.556
25	DIALEITVTNSPSNPR	1726.866	1726.897
26	SEDEVGSLIEYEFR	1672.764	1672.770
27	VESKGLEKVTCEPQK	1731.866	1731.895

28	REITEKQIDDNRK	1644.792	1644.866
29	FSLFAER	869.476	869.452
30	YQTLNCSVNVNCVNIR	1954.003	1953.927
31	LNYLDILMR	1150.644	1150.629
32	AFIDVTAAAENIR	1390.739	1390.733
33	LPNAGTQVR	955.523	955.532
34	VSVPQTDMRPEK	1386.727	1386.705
35	EPWPNSDPPFSFK	1547.730	1547.717
36	NVISLTEDVDEFR	1536.744	1536.754
37	TQDYPSVPTLVR	1375.718	1375.722
38	RGEVGIYQVQLR	1417.801	1417.791
39	ALEHVDGTHVCQLPEDQK	2075.965	2075.981
40	GNIHLKPSFSDGLK	1512.749	1512.817
41	MDAGIICDVCTCELQK	1928.901	1928.822
42	YEGQFCEYDNFQCPR	2012.795	2012.790
43	SCVQCQAWGTGEKKGR	1879.865	1879.890
44	DEDDDCTYSYTMEGDGAPGPNSTVL VHK	3103.229	3103.278
45	QEVEENLNEVYR	1521.779	1521.718
46	VAPGYYTLTADQDAR	1640.779	1640.791
47	VPLFIRPEDDDEK	1572.778	1572.790
48	DVVSFEQPEFSVSR	1625.758	1625.781
49	LLELQEVDSLLR	1427.760	1427.810
50	VCAYGAQGEGPYSSLVSCR	2060.883	2060.916
51	VLVDNPKNR	1054.644	1054.600

Materials and Methods.

Solubilisation of Tumour Tissue

Human colon cancer tissue expressing the A3 antigen was provided by hospitals in Sweden and stored frozen at -70°C in the tissue bank at ABR. Frozen colon cancer tissues were sliced with a scalpel and transferred into a tube containing cold isotonic sucrose buffer (0.25M sucrose, 10mM KCl, 1.5M $MgCl_2$, 50mM Tris-HCl pH 7.4 at 25°C) containing 1% (v/v) Nonidet P-40 (NP-40) and protease inhibitors (Completet™Protease Inhibitor Cocktail Tablet, Boehringer Mannheim). Tissue was homogenised with an Ultra-Turrax homogeniser and were left to solubilise at 0°C. The solubilised preparation was centrifuged at 11,000 rpm (Hettich centrifuge Universal 30 RF rotor), to remove cell debris. The supernatant was further centrifuged at 108,000g at 4°C

(Beckman Ultracentrifuge Ti-60 rotor), and finally filtered through a 0.2 µm Minisart plus filter (Sartoriuis AG Gottingen Germany).

Affinity Purification of tissue antigens

A3scFv-SEAm9 was coupled to a NHS-activated HiTrap® column (Pharmacia Biotech Uppsala Sweden), according to the manufacturer's recommendations. The control and pre-column were coupled with C215Fab-SEAm9, and the control, pre-column and column were set up in series. All columns were washed with pre-wash buffer (20mM Tris HCl pH7.5 at 4°C containing 0.2% NP 40). The extract was loaded onto the column at 0.1ml/min, and the flow through was recirculated. The columns were then washed with start buffer. Bound antigen was eluted in a pH gradient of diethylamine starting at pH 7.5 up to 11.0. 2.5 ml of eluant was collected and concentrated down to 75 µl. The purification was performed at 4°C using an AKTA FPLC system (Amersham Pharmacia Biotech Uppsala Sweden). Eluted protein was analysed by SDS PAGE and silver staining. Individual bands were excised, digested with trypsin and the masses of the peptide were determined using a MALDI-TOF instrument by Protana A/S (Odense, Denmark). The peptide masses were then compared in a computer search with all tryptic peptide masses for each protein in the SWISSPROT database, a service provided by Protana A/S (Odense Denmark).

EXAMPLE 6

A3scFv-SEAm9 detects a novel α6β4 integrin epitope

Commercial antibodies to human α6 integrin and β4 integrin were compared to A3 on normal and malignant colon sections. The reactivity, shown in Figure 6, demonstrates that A3 is restricted to the colon epithelium (Fig 6[i]), and malignant cell in the tumour (Fig 6 [ii]). Commercial antibody NKI-GoH3 to α6 integrin, also reacted with normal colon (Fig 6 [iii]) and colon cancer (Figure 6 [iv]). Reaction was seen in epithelial cells of colon and malignant cells (arrows)

but also in blood vessels (BV), some stromal components (s) and in muscularis mucosae (mm). The reaction observed with commercial ASC-3 anti-β4 integrin antibody was similar to that noted with anti-α6 antibody but weaker, in both normal colon (v) and colon cancer (vi).

Materials and Methods

Antibody

A3 scFv was selected from the M fascicularis library. The VH and VL genes from this were released by restriction enzyme digestion and fused to the Staphylococcal Enterotoxin AE chimeric mutant (D227A) to generate the A3scFv-SEAm9. This demonstrated very low levels of non-specific binding and allowed sensitive detection by secondary antibodies. ASC-3 anti-human-β4 integrin antibody and NKI-GoH3 anti-human-α6 integrin antibody were from Becton Dickinson (Copenhagen, Denmark)

Immunohistochemistry

Tumour and normal tissue samples were obtained from the Department of Surgery Lund Hospital. These were rate-frozen in iso-pentane, which had been pre-cooled in liquid nitrogen. Samples were stored at -70°C until sectioned. After cryosectioning the sections were air dried over night, fixed in cold acetone and blocked with avidin/biotin (Vector Burlingame CA). Primary antibody was then added to the section for one hour.

The secondary antibodies were incubated for 30 minutes followed by streptavidin-biotin/HRP (Dakopatts Copenhagen Denmark) for a further 30 minutes. Extensive washing was perfromed between all these steps with 50mM Tris pH 7.6, 0.15M NaCl. Diaminobenzidine (DAB) was used as chromogen and the sections were counterstained in 0.5% methyl green. Controls included a non-tissue reactive Fab and SEA-D227A or no primary antibody. All antibodies were used at a final concentration of 5 µg/ml. Results were expressed as negative, weak, moderate or strong staining.

EXAMPLE 7

The A3 Tumour Associated Antigen reacted with α6 and β4 integrin antibodies in a capture ELISA

Crude tumor extract or A3 antigen purified by A3-affinity chromatography (see example 5) was analysed by a capture ELISA. Commercial antibodiy ASC-3 specific for beta 4 integrin were used as capture antibody, to which different dilutions of crude tumor extract was applied. This was then chased with A3scFv-SEAm9. Bound A3scFv-SEAm9 was then detected with anti-SEA-HRP (Fig 7A). In Figure 7B the commercial anti-α6 integrin antibody NKI-GoH3 was used to capture different dilutions of the concentrated A3-affinity purified eluate. In a similar way as in Figure 7A the captured proteins were chased with A3scFv-SEAm9 and detected with anti-SEA-HRP. In both experiments a concentration dependent signal was detected. These results confirm the specificity of A3 to α6β4 intergin heterodimer, which was also shown to be specifically isolated from the A3-affinity column in example 5.

Material and Methods

Commercial antibodies NKI-GoH3 or ASC-3 (Becton Dickinson Copenhagen Denmark) 100 µl, were used to coat the well of an E.I.A./R.I.A.-plate (Costar) in 0.05 M $NaHCO_3$, pH 9.6. The reaction was allowed to continue overnight at 4°C, after which the plates were washed 4 times in DPBS + 0.05 % Tween 20. Wells were then blocked with 200 µl 3 % non-fat milk powder in DPBS + 0.05 % Tween 20, for 1-2 h at room temperature (RT) with shaking. Wells were again washed as above and 100 µl antigen extract diluted in 3 % non-fat milk powder in DPBS + 0.05 % Tween 20, was applied for 2 h at RT with shaking. Wells were again washed (4 x DPBS + 0.05 % Tween 20) after which 100 µl of the primary antibody diluted in 3 % non-fat milk powder in DPBS + 0.05 % Tween 20 was incuabted for 2 h at RT with shaking. Wells were washed again as above and 100 µl of the secondary antibody

diluted in 3 % non-fat milkpowder in DPBS + 0.05 % Tween 20 was added to each well for 1 h at RT with shaking. Wells were again washed as above and colour developed by the addition of 100 µl peroxidase substrate (Sigma Fast OPD Peroxidase Substrate Tablet Set P-9187). The reaction was allowed to continue for 30 min at RT, in the dark and shaking before the reaction was stopped by the addition of 50 µl 3 M H_2SO_4. The absorbance was read at 490 nm.

EXAMPLE 8

Western Blot analysis of the A3 tumour antigen

A3-affinity purified tumour antigen extracts were separated by SDS-PAGE and transferred to membranes for Western blot analysis. Extracts were applied directly or heated to 100°C for 5 minutes or heated to 100°C for 5 minutes but in the presence of mercaptoethanol (BME) (Figure 8). The membranes were then probed with A3scFv-SEAm9 and anti-SEA-HRP or anti-human-α6 integrin or anti-human-β4 integrin antibodies. The anti-β4 integrin antibody did not react with any protein on the membrane (Fig 8[ii]). The anti-human-α6 integrin reacted with a major specie with apparent molecular weight between 90 - 140 kDa in the A3-affinity purified tumour antigen extract (Figure 8[iii]). The same species was also detected by A3scFv-SEAm9, which also was detected after heating but was much weaker under reduced conditions (with BME present) (Figure 8[i]). The major band detected in the 90 - 140 kDa interval corresponds to the bands in example 5, that were analysed by peptide mapping and were found to contain α6 integrin and β4 integrin.

Materials and Methods

ASC-3 anti-human-β4 integrin antibody and NKI-GoH3 anti-human-α6 integrin antibody were from Becton Dickinson (Copenhagen, Denmark). Samples were resolved by SDS-PAGE in 0.25M tris-glycine pH 8.9 and 0.1%SDS at 100V through the upper gel, then 170V through the resolving gel.

Molecular weight standards (Biorad broad Range, Biorad) were included on all gels. Resolved samples were transferred to nitrocellulose (Biorad) in transfer buffer (10 mM Tris base, 2M glycine, 40% (v/v) methanol) at 100V for 1 hour. Membranes were blocked with 5% (w/v) BSA/TBS for at least 2 hours at 4°C, then incubated with the appropriate antibody diluted in 5% BSA/TBS/0.2% azide. This reaction was allowed to proceed for at least 2 hours at RT, after which the membrane was washed extensively in TBST-T. Bound antibody was detected by incubation of membranes for 1 hour with HRP conjugated antibody diluted in TSB-T containing 5% milk powder. Membranes were then incubated with enhanced chemiluminescence (ECL) detection reagents (Renaissance® NEN™ Life Science Products, Boston MA) for 1 minute and exposed to film for up to 1 hour.

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LEGENDS TO FIGURES

Figure 1 *The A3 tumour-associated antigen is homogeneously expressed in primary and metastatic tumours*

Immunohistochemical staining of frozen and acetone fixed sections of human tumour tissues using A3 scFv-SEA(D227A) and C215 Fab-SEA(D227A) at 70 nM. The A3 scFv fusion protein reacted strongly and homogeneously with both primary colon and pancreatic carcinoma resected from tumour patients. A representative staining of a primary colon cancer is shown for C215 Fab-SEA(D227A) in (A) and for A3 scFv-SEA(D227A) in (B). Staining by A3 scFv-SEA(D227A) of a colon cancer liver metastasis is shown in (C) and of a primary pancreatic cancer in (D).

Figure 2 *A3 scFv-SEA(D227A) coated Colo205 tumour cells are efficiently killed by T cells.*

Superantigen antibody dependent cellular cytotoxicity (SADCC) towards Colo205 cells mediated by A3 scFv-SEA(D227A) reached the same maximal cytotoxicity as the anti-Ep-CAM fusion protein C215 Fab-SEA(D227A), although at a ten-fold higher concentration. The absence of cytotoxicity mediated by the D1.3 scFv-SEA(D227A) demonstrates the need of a tumour targeting antibody moiety in the fusion protein.

Figure 3

Immunoaffinity chromatography of tumor extract on a A3scFv-SEAm9 coupled column. Protein bound to A3 coupled columns was washed extensively then eluted as described in Materials and Methods in Example 5. The eluted fractions were examined by UV spectroscopy (arrow) and a single peak identified. The sample was eluted with a pH gradient as indicated by an x.

Figure 4

A3 antigen preparation was separated on a non-reduced SDS PAGE and silv r-stained. Previous Western analysis had defined a molecular weight range in which the A3 antigen was believed to reside. The bands evident

within this region (Labelled I and II) were excised for peptide mapping analysis

Figure 5A and 5B

Epithelial integrin α6β4: complete primary structure of α6 and variant forms of β4 (precursor) (Tamura et al J Cell Biol 111:1593-1604 (1990)). The matched peptides shown in SEQ ID NOs: 5-51 are underlined in the sequences of human α6 (Fig 5A) integrin and β4 (precursor) (Fig 5B) integrin as published.

Figure 6

Immunohistochemistry of normal and malignant colon using A3scFv and commercial anti-human α6 and β4 integrin monoclonal antibodies.

Figure 7A and 7B

Capture ELISA. In fig 7A monoclonal antibody ASC-3 specific for β4 integrin was used as capture antibody, to which different dilutions of crude tumor extract was applied. In fig 7B the anti-α6 integrin monoclonal antibody NKI-GoH3 was used to capture different dilutions of the concentrated A3-affinity purified eluate. In both fig 7A and 7B the captured integrin antigen was then successfully detected with A3scFv-SEAm9.

Figure 8A and 8B

Western blot analysis of the eluate from the A3-affinity column. The primary antibodies used are (i) and (ii) A3scFv-SEAm9, (iii) ASC-3 anti-human-β4 integrin antibody and (iv) NKI-GoH3 anti-human-α6 integrin antibody. Lane A - the eluate was applied directly, lane B - the eluate was heated to 100°C for 5 minutes, and lane C - the eluate was heated to 100°C for 5 minutes but in the presence of mercaptoethanol. Positions of molecular weight standards are indicated.

CLAIMS

1. An antibody, or a derivative or a fragment thereof, having a binding structure for a target structure displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells, said binding structure comprising the complementarity determining region (CDR) sequences in the light chain comprising essentially the amino acids number 23-33 (CDR1), 49-55 (CDR2), 88-98 (CDR3) of the amino acid sequence shown in SEQ ID NO:2, and the CDR sequences in the heavy chain comprising essentially the amino acids number 158-162 (CDR1), 177-193 (CDR2), 226-238 (CDR3) of the amino acid sequence shown in SEQ ID NO:2, or other binding structures with similar unique binding properties.

2. An antibody according to claim 1, which is phage selected.

3. An antibody according to claim 1, wherein the sequences are of Macaca fascicularis origin.

4. A derivative of an antibody according to claim 1, which is of human origin.

5. An antibody according to claim 1, wherein the sequences have an identity of at least 84% to corresponding sequences of human origin.

6. An antibody according to claim 1, which has low immunogenicity or non-immunogenicity in humans.

7. An antibody according to claim 1, which has been derivatised by genetically linking to other polypeptides, and/or by chemical conjugation to organic or non-organic chemical molecules, and/or by di-, oligo- or multimerisation.

8. An antibody according to claim 1, which is genetically linked or chemically conjugated to cytotoxic

polypeptides or to cytotoxic organic or non-organic chemical molecules.

9. An antibody according to claim 1, which is genetically linked or chemically conjugated to biologically active molecules.

10. An antibody according to claim 1, which is genetically linked or chemically conjugated to immune activating molecules.

11. An antibody according to claim 1, which has been changed to increase or decrease the avidity and/or affinity thereof.

12. An antibody according to claim 1, which has been changed to increase the production yield thereof.

13. An antibody according to claim 1, which has been changed to influence the pharmacokinetic properties thereof.

14. An antibody according to claim 1, which has been changed to give new pharmacokinetic properties thereto.

15. An antibody according to claim 1, which is labeled and the binding thereof is inhibited by an unlabeled form of said antibody and not by other binding structures, and not inhibiting the binding of other binding structures having other binding specificities.

16. An antibody according to claim 1, wherein said binding structure recognises a non-reduced form of $\alpha 6\beta 4$ integrin.

17. A target structure displayed in, or on the surface of, tumour cells, said target structure

a) having the ability of being specifically blocked by and to specifically block the binding structure of an antibody as defined in any one of claims 1-14, and other binding structures with similar binding properties,

b) being displayed in, and on the surface of, human gastrointestinal epithelial cells,

c) having substantial homology with $\alpha 6$ and/or $\beta 4$ integrin chains or variants thereof, representing a shared or unique epitope,

d) being highly expressed on the surface of tumour cells, and

e) being a target for cytotoxic effector mechanisms.

18. A target structure according to claim 17, wherein the binding structure is labeled and the binding thereof is inhibited by an unlabeled form of said binding structure and not by other binding structures, and not inhibiting the binding of other binding structures having other binding specificities.

19. A target structure according to claim 17, wherein said binding structure comprises one or more of the complementarity determining region (CDR) sequences comprising essentially the amino acids number 23-33, 49-55, 88-98, 158-162, 177-193, 226-238 of the amino acid sequence shown in SEQ ID NO:2, or other binding structures with similar unique binding properties.

20. A target sturcture according to claim 17, wherein said binding structure is an antibody.

21. A target structure according to claim 20, wherein said antibody comprises the variable region of a light chain comprising essentially the amino acids number 1-109 of the amino acid sequence shown in SEQ ID NO:2, and the variable region of a heavy chain comprising essentially the amino acids number 128-249 of the amino acid sequence shown in SEQ ID NO: 2.

22. A target structure according to any one of claims 17-21, which is expressed homogenously in human colonic epithelial cells and less in pancreatic duct and bile duct cells.

23. A target structure according to any one of claims 17-22, the expression of which is correlated to gastrointestinal epithelial differentiation.

24. A target structure according to any one of claims 17-23, which comprises essentially the amino acid sequence of α6 integrin shown in SEQ ID NO: 3 and/or of β4 integrin shown in SEQ ID NO: 4, and/or one or more

fragments, and/or variants or splice variants, and or subunits, thereof.

25. A target structure according to claim 24, which comprises homo- or hetero-monomers or homo- or hetero-multimers of said α6β4 integrin and/or of said one or more fragments and/or variants and/or subunits thereof.

26. A target structure according to claim 24, which has an apparent molecular weight in its non-reduced form of from 90 to 140 kDa, most preferred from 80 to 160 kDa.

27. A target structure according to claim 24, which comprises a peptide or polypeptide(s) comprising essentially any one of the amino acid sequences shown in SEQ ID NOs: 5-51, or comprises a molecule complexed to said polypeptide(s).

28. A target structure according to any one of claims 24-27 recognised, exclusively or not, in its non-reduced form by the binding structure comprised by the antibody as defined in any one of claims 1-16.

29. A substance which binds to the target structure as defined in any one of claims 17-28, which substance is an organic chemical molecule or a peptide.

30. A substance, which is an anti-idiotype of a binding structure to said target structure as defined in anyone of claims 17-28.

31. A substance according to claim 30, which anti-idiotype is specifically blocked by and specifically blocks a binding structure having binding specificity for said target structure.

32. A substance which blocks the function of the target structure as defined in any one of claims 17-28, which substance is an organic chemical molecule or a peptide.

33. A binding structure which recognizes a target structure as defined in any one of claims 17-28, and which is of an organic chemical nature.

34. A pharmaceutical composition comprising as an active principle an antibody as defined in any one of claims 1-16.

35. A pharmaceutical composition comprising as an active principle a target structure as defined in any one of claims 17-28.

36. A pharmaceutical composition comprising as an active principle a substance as defined in any one of claims 29-32.

37. A vaccin composition comprising as an active principle an antibody as defined in any one of claims 1-16, or a target structure as defined in any one of claims 17-28, or a substance as defined in any one of claims 29-32.

38. A method of therapy for treating conditions based on an anti-angiogenic mechanism, whereby an antibody as defined in any one of claims 1-16, or a target structure as defined in any one of claims 17-28, or a substance as defined in any one of claims 29-32, is administered to a human subject.

39. A method of treating human metastatic diseases, wherein an antibody as defined in any one of claims 1-16 is administered to a human subject.

40. A method of in vitro histopathological diagnosis and prognosis of human malignant desease, whereby a sample is contacted with an antibody as defined in any one of claims 1-17 and an indicator.

41. A method according to claim 40, which method comprises tumour typing.

42. A method according to claim 40, which method comprises tumour screening.

43. A method according to claim 40, which method comprises tumour diagnosis and prognosis.

44. A method according to claim 40, which method comprises monitoring premalignant conditions.

45. A method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily

fluids of an antigen comprising a target structure, as defined in any one of claims 17-28,

46. A method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of an antibody as defined in any one of claims 1-16 is assayed.

47. A method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of a complex of a) an antigen comprising a target structure, as defined in any one of claims 17-28, or a structure, as defined in any one of claims 29-32, is assayed, and b) an antibody, as defined in any one of claims 1-16, is assayed.

48. A method for in vivo diagnosis and prognosis of human malignant disease, whereby the localisation of an antibody, as defined in any one of claims 1-16, to tumour deposits in a human subject is determined.

49. A method according to claim 48, whereby said antibody is administered to the subject before the determination.

50. A method according to claim 48, whereby said antibody is accumulated in tumour deposits.

51. A method according to any one of claims 48-50, which is quantitative.

52. A method for therapy of human malignant disease, whereby an antibody, as defined in any one of claims 1-16, is administered to a human subject.

53. A method according to claim 52, whereby said antibody has been changed by being genetically linked to molecules giving the combined molecule changed pharmacokinetic properties.

54. A method according to claim 52, whereby said antibody has been changed by being derivatised.

FIG. 1

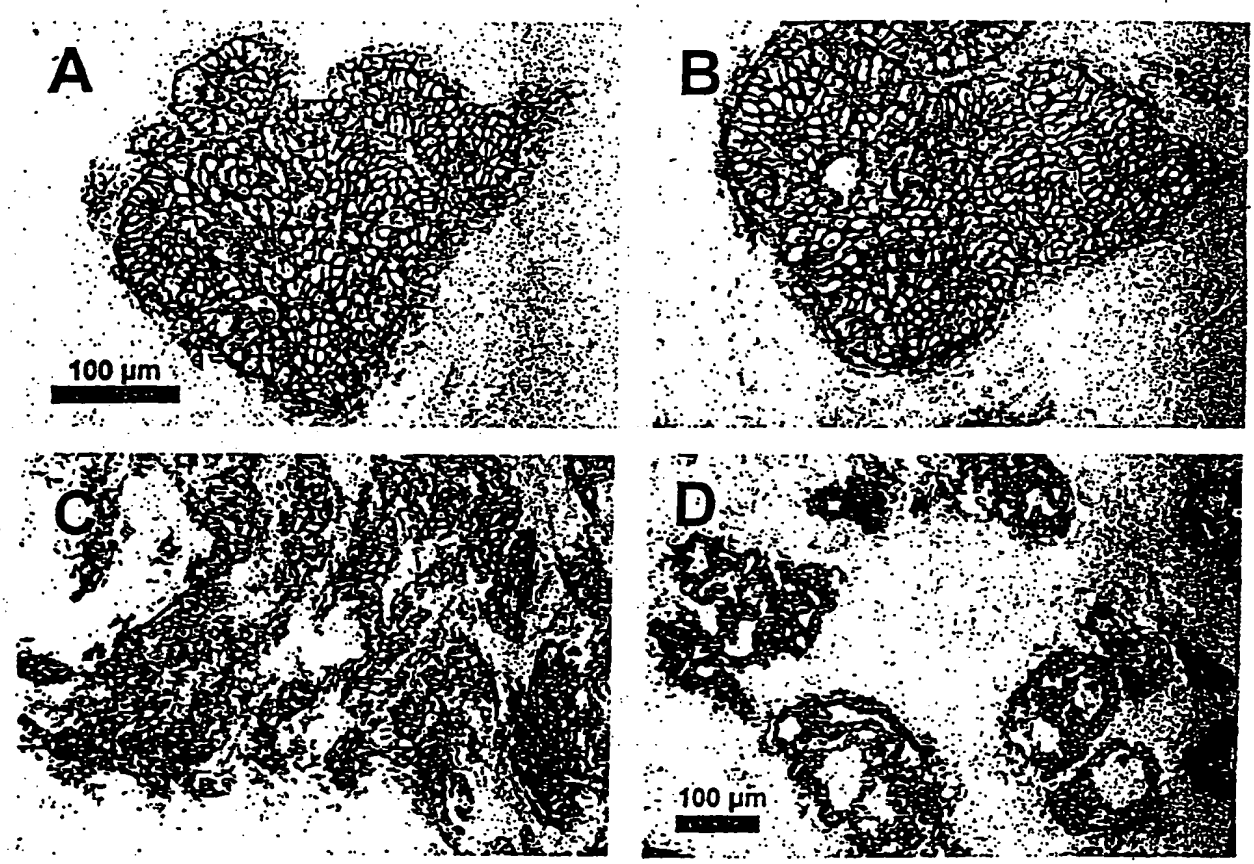
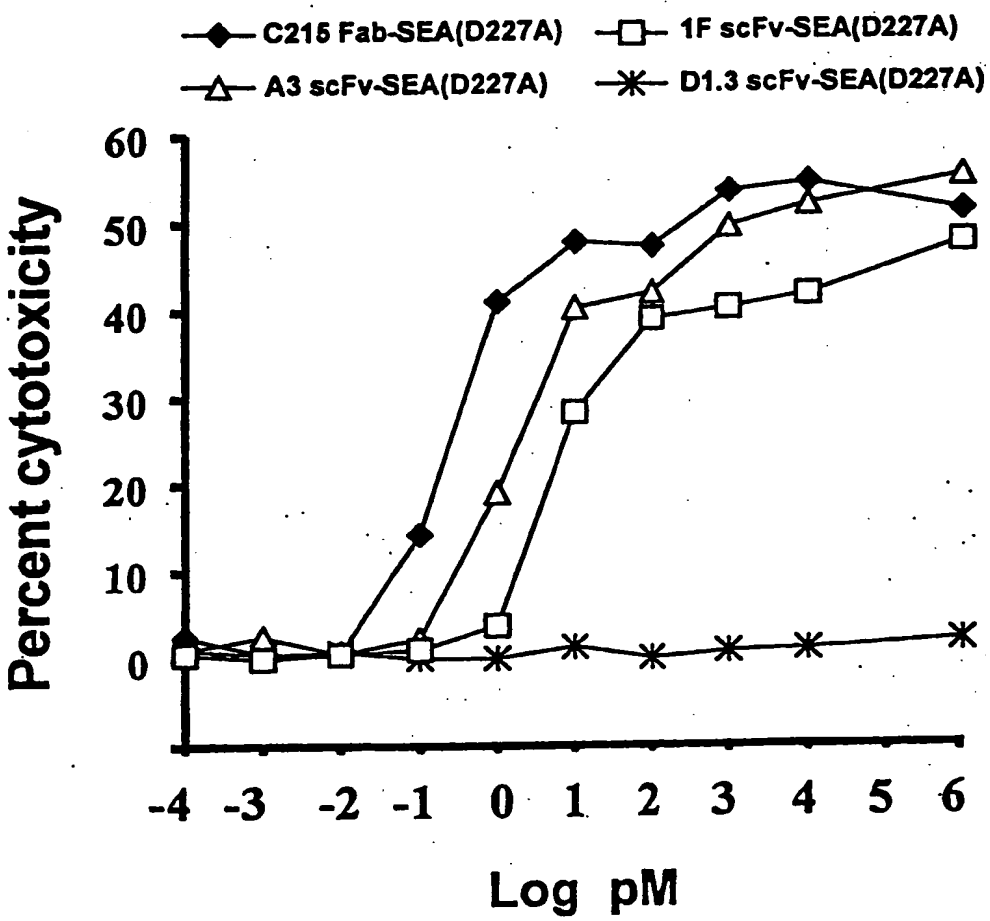


FIG. 2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30
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10
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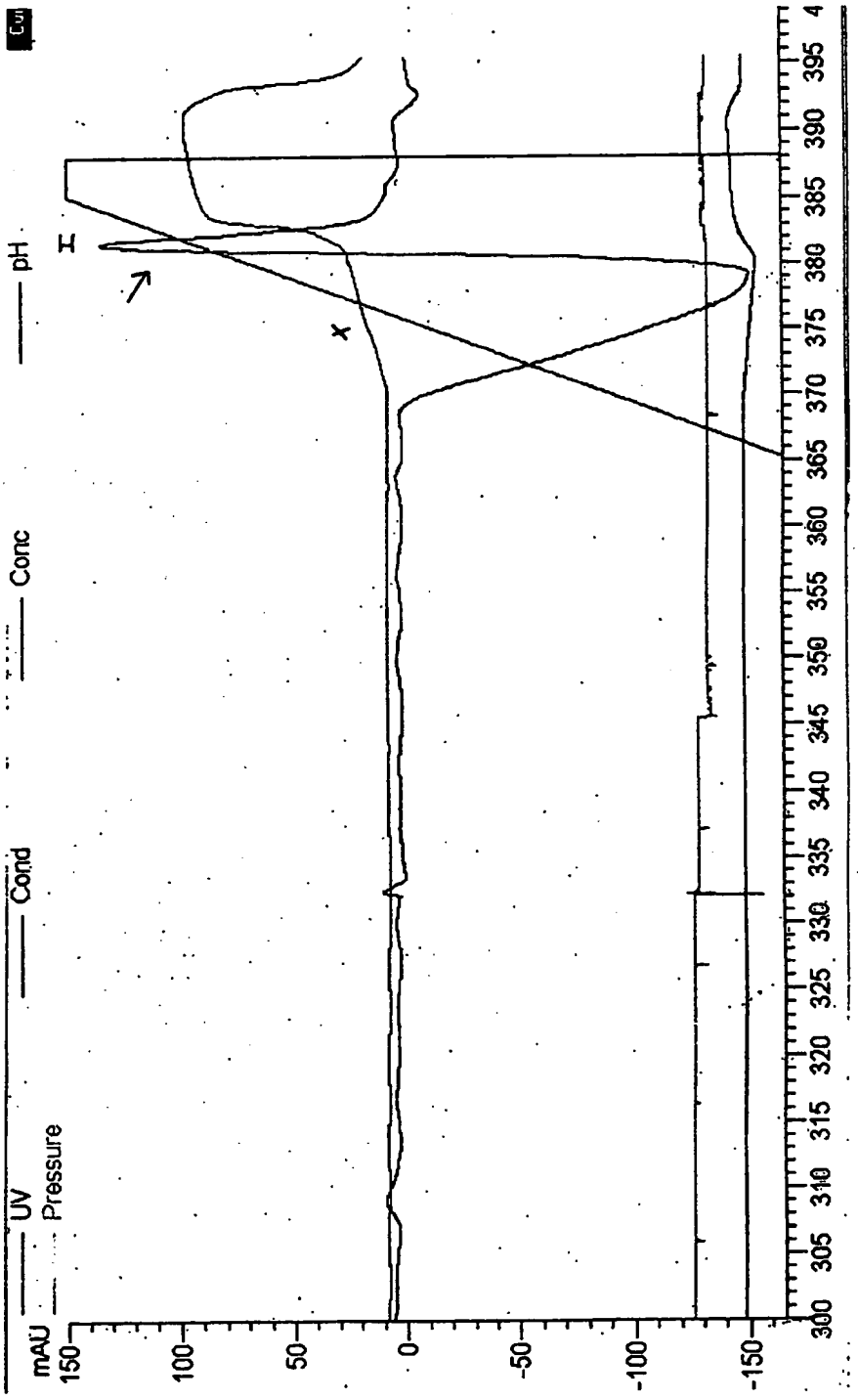
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FIG. 3

FIG. 4

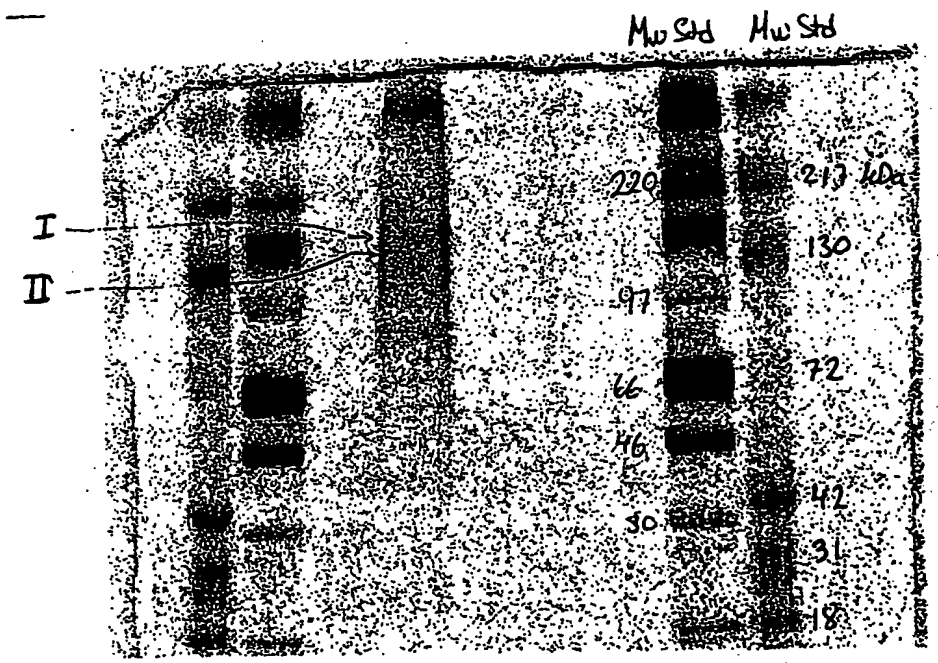

FIG. 5A

TA6-Human integrin ALPHA-6A

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WMGVTVQSQGPGGKVVTCAHRYEKRQHVNTKQESRDIFGRCYVLSQNLRIEDDMD
GGDWSFCDGRLRGHEKFGSCQQGVAATFTKDFHYIVFGAPGTYNWKGIVRVEQKN
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QYFDRDGEVGGAVYVYMNQQGRWNNVKPIRLNGTKDSMFGIAVKNIGDINQDGYP
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AVGSLSDSVTIFRSRPVINIQKTITVTPNRIDLRQKTACGAPSGICLQVKSCFEYTANPA
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DDNVCNSNLKLEYKFCTREGNQDKFSYLPIQKGVPELVLKDQKDIALEITVTNSPSNP
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FKRNSNVTFYLVLSTTEVTFDTPDLDINLKLETTSNQDNLAPITAKAKVVIELLLSVSG
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FAERKYQTLNCSVNVNCVNIRCPLRGLDSKASLILRSRLWNSTFLEEYSKLNYLDILM
RAFIDVTAAAENIRLPNAGTQVRVTVFPSKTVAQYSGVPWWIILVAILAGILMLALLV
FILWKCGFFKRNKKDHYDATYHKAEIHAQPSDKERLTSDA

FIG. 5B

INTEGRIN BETA-4 (PRECURSOR)

MAGPRPSPWARLLLAALISVSLSGTLANRCKKAPVKSCTECVRVDKDCAYCTDEMF
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IVGYLVTCEMAQGGGPATAFRVDGDSPESRLTVPGLSENVPYKFKVQARTTEGFGPE
REGIITIESQDGGPFPQLGSRAGLFQHPLQSEYSSITTTHTSATEPFLVDGPTLGAQHLE
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FIG. 6

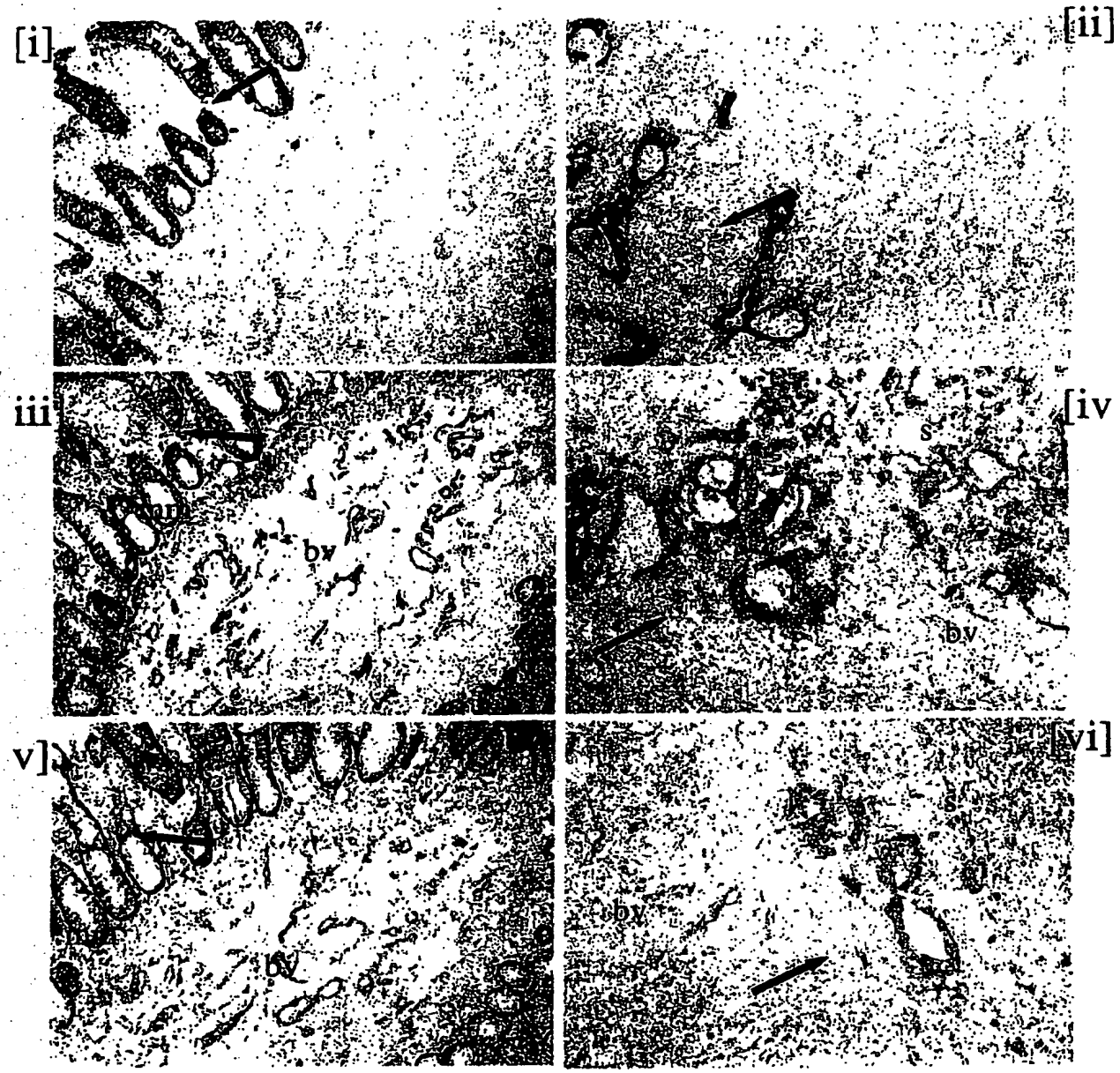


FIG. 7A

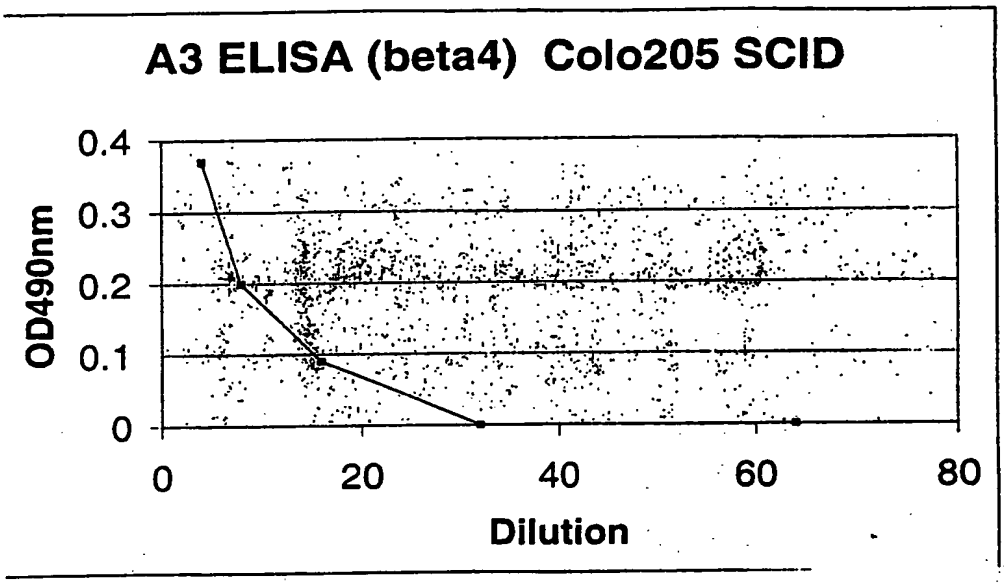


FIG. 7B

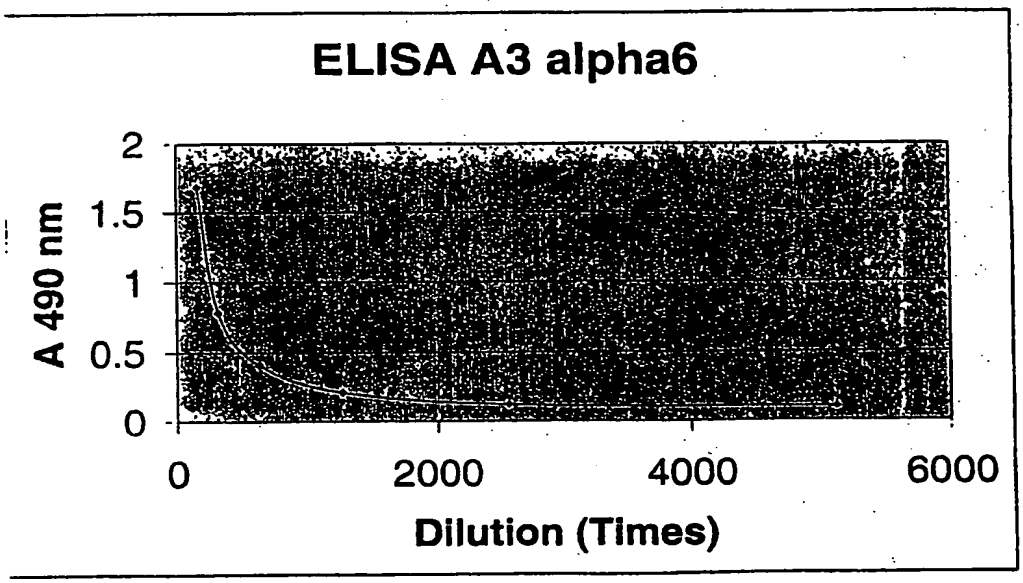
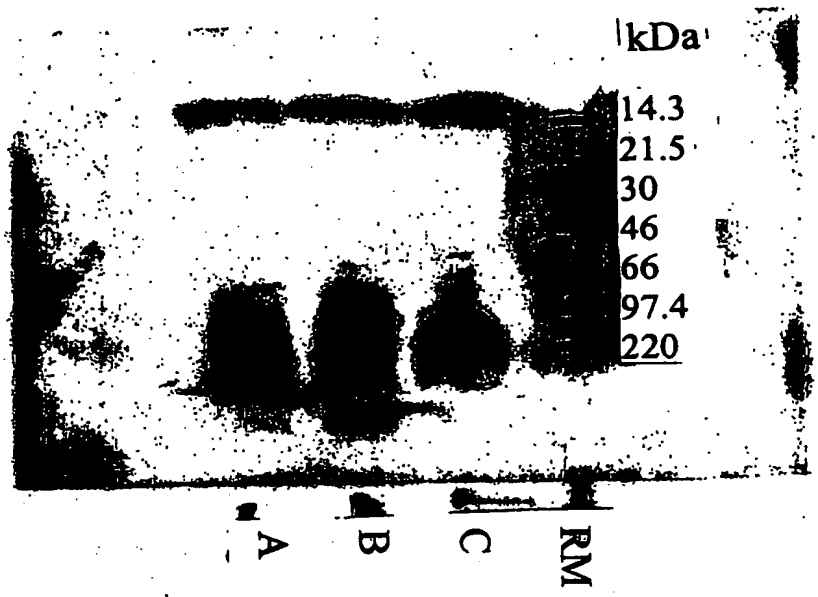


FIG. 8A
(i)

scFvA3FabSEAm9 (4 µg/ml)

αSEA-HRP (1/2000)



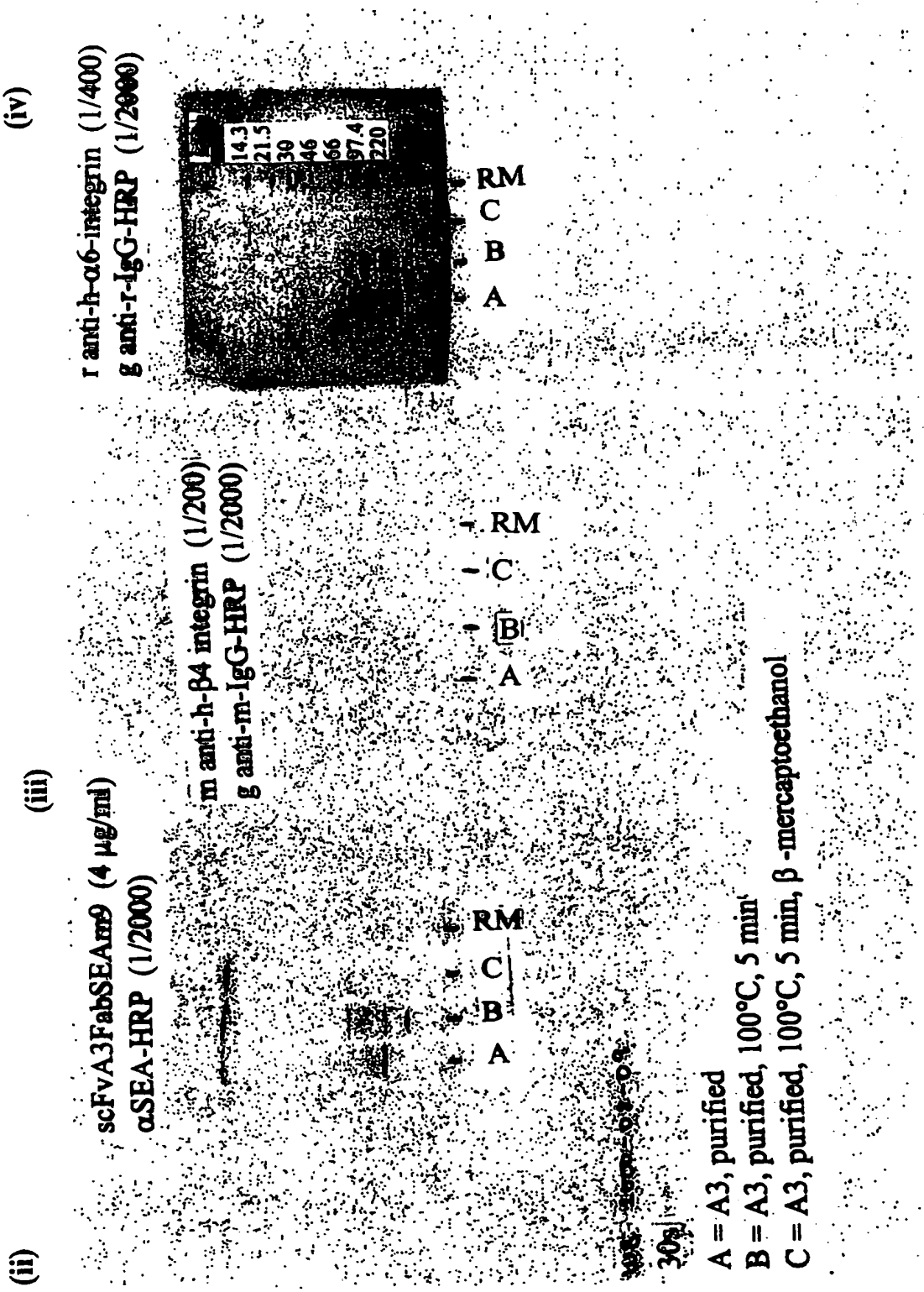


FIG. 8B

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aca gtc agg atg acc tgc caa gga gac agc ctc aaa acc tat tat gca   96
Thr Val Arg Met Thr Cys Gln Gly Asp Ser Leu Lys Thr Tyr Tyr Ala
            20                  25                  30

agc tgg tac cag cag aag cca ggc cag gtc cct gtg ctg gtc atc tat   144
Ser Trp Tyr Gln Gln Lys Pro Gly Gln Val Pro Val Leu Val Ile Tyr
        35                  40                  45

ggt aac aac tac cgg ccc tca ggg atc cca ggc cga ttc tct ggc tcc   192
Gly Asn Asn Tyr Arg Pro Ser Gly Ile Pro Gly Arg Phe Ser Gly Ser
    50                  55                  60

tgg tca gga aac aca gct tcc ttg acc atc act gcg gct cag gtg gaa   240
Trp Ser Gly Asn Thr Ala Ser Leu Thr Ile Thr Ala Ala Gln Val Glu
65                  70                  75                  80

gat gag gct gac tat tac tgt aac tcc tgg gac agc agc ggt acc cat   288
Asp Glu Ala Asp Tyr Tyr Cys Asn Ser Trp Asp Ser Ser Gly Thr His
                85                  90                  95

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Pro Val Phe Gly Gly Gly Thr Arg Val Thr Val Leu Gly Gln Ala Asn
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